Neuron, Vol. 39, 1019–1029, September 11, 2003, Copyright ©2003 by Cell Press

A Putative *Drosophila* Pheromone Receptor Expressed in Male-Specific Taste Neurons Is Required for Efficient Courtship

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Summary

Propagation in higher animals requires the efficient and accurate display of innate mating behaviors. In *Drosophila melanogaster*, male courtship consists of a stereotypic sequence of behaviors involving multiple sensory modalities, such as vision, audition, and chemosensation. For example, taste bristles located in the male forelegs and the labial palps are thought to recognize nonvolatile pheromones secreted by the female. Here, we report the identification of the putative pheromone receptor GR68a, which is expressed in chemosensory neurons of about 20 male-specific gustatory bristles in the forelegs. *Gr68a* expression is dependent on the sex determination gene *doublesex*, which controls many aspects of sexual differentiation and is necessary for normal courtship behavior. Tetanus toxin-mediated inactivation of *Gr68a*-expressing neurons or transgene-mediated RNA interference of *Gr68a RNA* leads to a significant reduction in male courtship performance, suggesting that GR68a protein is an essential component of pheromone-driven courtship behavior in *Drosophila*.

Introduction

Courtship behaviors are highly diversified, innate behaviors essential for propagation in higher animals. In general, courtship is composed of a series of behavioral displays controlled by the CNS, modulated by the endocrine system, and triggered only by highly specific, external stimuli that emanate from the mating object.

In wild *Drosophila melanogaster*, mating occurs near feeding sites to which they are attracted by long-range olfactory cues (Markow, 1988). Mate recognition, courtship, and mating are then mediated by visual, auditory, and pheromone signals and displayed in a stereotypic sequence of behaviors particularly well defined in the male (Greenspan, 1995; Hall, 1994): first, the male orients toward and follows a female (1), taps her abdomen with his forelegs (2), and proceeds to generate a "courtship song" by rapid wing vibrations (3). He then licks the female's genitalia (4), curls his abdomen to attempt mounting (5), and eventually succeeds in mounting and copulation with the female (6). These steps entail visual (1) and chemosensory (2 and 4) recognition of female features by the male, auditory reception of the male courtship song by the female (3), and somatosensory agility of both sexes (3, 5, and 6). Even though this behavior is displayed in a stereotypical, sequential manner, a male generally executes each step multiple times before proceeding to the next. Females do not display a distinct courting behavior, but mated females actively reject a new potential mate by walking away, kicking with her hind legs, flicking of her wings, and extension of her ovipositor (Hihara, 1981; Spieth and Ringo, 1983).

Efficient performance of courtship is the major determinant of mating success and can be quantified in single pair mating experiments by measuring mating latency (time from a first encounter between a male and a female until copulation), nonmating frequency, or the courtship index (CI = the percentage of time a male performs any of the first five courtship steps during a mating experiment). All these parameters have proved valuable for the quantification of male mating performance (Hall, 1994; Villella and Hall, 1996; Waterbury et al., 1999).

Mating behavior and sexual differentiation are regulated by a genetic cascade of splicing factors including Transformer (TRA) and Transformer2 (TRA2) that control the sex-specific, alternative splicing of mRNAs encoding the transcription factors Doublesex (DSX) and Fruitless (FRU) (Cline and Meyer, 1996; MacDougall et al., 1995). $DSX^{m\ (male)}$, $DSX^{f\ (female)}$, and FRU^{m} control the expression of numerous male and female effector genes responsible for differentiation and maintenance of sexual identity. Several *dsx*-dependent effectors have been identified and were found to be expressed in endocrine tissues of the adult (Burtis et al., 1991; Dauwalder et al., 2002; Fujii and Amrein, 2002) or in the genital disc during differentiation of adult structures (Ahmad and Baker, 2002). FRU^{m} and DSX^{m} are required, but neither alone is sufficient, for wild-type male courtship behavior, because males lacking FRU^{m} but expressing DSX^{m} (*X/Y; fru^{1}/ fru^{1}*) and intersexes which lack DSX^{m} but express FRU^{m} (*X/Y dsx^{1}/Df(dsx)*) display severely reduced courtship behavior (Ryner et al., 1996; Villella et al., 1997; Villella and Hall, 1996). Additionally, *fru* males court both males and females indiscriminately (Gailey and Hall, 1989).

Several lines of evidence suggest that pheromone-elicited mate recognition is mediated mainly through the contact chemosensory system. For example, males tap the pheromone-coated, female abdomen and genitalia with their forelegs and labial palps, respectively (Hall, 1994), both of which are covered with taste bristles (Hall, 1994; Stocker, 1994). The taste bristles on the forelegs are also implicated in a sex-specific function due to a quantitative difference in their number between males (~50) and females (~37) (Nayak and Singh, 1983; Stocker, 1994; Meunier et al., 2000). Finally, the chemical properties of the known female pheromones, which are nonvolatile, long-chain hydrocarbons (Coyne et al., 1994; Ferveur et al., 1996), further support a role for contact chemosensory neurons/receptors in male courtship behavior.

Taste bristles in the labial palps and legs are composed of two to four gustatory receptor neurons (GRNs). We and others have recently identified a family of about 70 G protein-coupled receptor (GPCR) genes, members

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of which are expressed in small subsets of GRNs in all known taste organs including the labial palps and the forelegs. (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001). Upon analysis of about one quarter of the *Gr* genes, we now report the expression and function of *Gr68a*, a *Gr* gene expressed in chemosensory neurons of about ten male-specific taste bristles in the foreleg. We propose that GR68a recognizes a female pheromone(s) involved in the second step of the courtship display, which is essential for efficient execution of the entire courtship sequence and timely mating.

Results

To better understand the possibly diverse functions of different GR proteins, we are in the process of visualizing the global expression profile of all *Gr* genes. The widely dispersed location of taste bristles, low expression level of *Gr* genes, and intractability of many taste organs (legs and wings) to in situ hybridization methods (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001) made it necessary to utilize the GAL4/UAS system (Brand and Perrimon, 1993) to determine the spatial distribution of GRNs expressing a given receptor. Specifically, the yeast transcriptional activator GAL4 was expressed under the control of the putative promoter of various *Gr* genes, and GAL4 activity was visualized with UAS-*lacZ* or UAS-*gfp* reporter genes (Dunipace et al., 2001; Scott et al., 2001). Initial investigations using this method revealed that most *Gr* genes are expressed in a small fraction of chemosensory neurons in spatially defined and distinct sets of GRNs in a subset of taste organs (labelum, pharyngeal sense organs, legs, and wings (Dunipace et al., 2001; Scott et al., 2001).

Gr68a Is Expressed in Gustatory Neurons of the Male Foreleg

Upon analysis of about a quarter of the 70 *Gr* genes, we identified a *Gr* gene, *Gr68a*, exhibiting the hallmarks of a putative pheromone receptor. In adults, *Gr68a* is exclusively expressed in neurons of about ten male-specific taste bristles in the forelegs. No expression was observed in females or any other organ or structure of males (Figure 1A and data not shown). Identical β-gal or GFP expression patterns were observed with four independent transgenic *p[Gr68a]-Gal4* driver lines, indicating that male-specific expression reflected an intrinsic property of the *Gr68a* promoter (Figure 1A, Table 1, and data not shown). To verify that the β-gal-positive cells were indeed neurons and not support cells associated with taste bristles, we performed antibody staining and found that β-gal immunoreactive cells have the typical structure of sensory neurons and express ELAV protein, a pan-neuronal marker not expressed in other cell types (Figure 1B). To verify that the *Gr68a* gene is expressed in one of the chemosensory neurons and not in the single mechanosensory neuron present in taste bristles, we analyzed its expression in a *pox-neuro* (*poxn*) mutant background. POXN is necessary for specification of chemosensory neurons, and *poxn* mutant flies show a complete transformation of all chemosensory neurons into mechanosensory neurons (Nottebohm et al., 1994). Indeed, the *p[Gr68a]-Gal4* driver was not expressed in these flies (Figure 1C) which confirmed that *Gr68a* is expressed in chemosensory neurons in the male foreleg.

If *Gr68a* encodes a male-specific pheromone receptor, we predicted that the sex determination genes, which control all aspects of sexual differentiation, would regulate its expression. Thus, we investigated *Gr68a* expression in chromosomally female (XX) flies that were sexually transformed into Ψ males by mutations in *tra2* or *dsx* (for details see Experimental Procedures). Both types of Ψ males showed the normal male expression pattern of the *p[Gr68a]-Gal4* driver (Figure 1D). Since sex-specific *fru* expression is directly controlled by TRA and TRA2 (Heinrichs et al., 1998; Ryner et al., 1996), and hence, independent of *dsx* (i.e., *XX; dsx* Ψ males express no FRU^m), male-specific expression of *Gr68a* is *fru* independent. Thus, *Gr68a* is a *dsx*-dependent effector gene expressed in chemosensory neurons of taste bristles in the foreleg, which is consistent with a function for this gene in pheromone recognition.

Neurons Expressing *Gr68a* Are Required for Normal Courtship

To determine whether *Gr68a*-expressing neurons function in pheromone recognition, we inactivated neuronal transmission using tetanus toxin light chain protein (TNT) and investigated courtship and several other behaviors of such males. TNT, which cleaves the synaptic vesicle protein N-SYB, a protein essential for neurotransmitter release (DiAntonio et al., 1993), has been widely used in *Drosophila* to inactivate various types of sensory neurons, including chemosensory neurons in developing and adult *Drosophila* (Martin et al., 2002). We inactivated *Gr68a*-expressing neurons in males using a *UAS-tnt* reporter gene expressed under the control of the *p[Gr68a]-Gal4* driver (Sweeney et al., 1995). In addition, we generated males expressing an inactive TNT protein (TNT^{in}) in these neurons (Sweeney et al., 1995), to control for nonspecific effects of overexpression of GAL4 and TNT. It should be noted that TNT does not kill the neurons in these males, as coexpression of GFP persists for at least 18 days without apparent change in cell morphology or loss in fluorescence (data not shown). Overall sensory perception was not affected as two courtship-unrelated behaviors, sugar recognition/sensitivity and gravity locomotion, were not affected in males without functional *Gr68a*-expressing neurons (Table 2; for details see Experimental Procedures).

We next investigated the function of *Gr68a*-expressing neurons as putative pheromone receptor neurons. In principle, these neurons may be activated by a stimulatory female pheromone or an inhibitory male pheromone (Ferveur and Sureau, 1996). Males lacking functional GRNs expressing a stimulatory pheromone receptor may fail to recognize a female and therefore exhibit reduced courtship; alternatively, absence of GRNs expressing an inhibitory male pheromone receptor may lead to anomalous male-to-male courtship. We first investigated a stimulatory function of *Gr68a*-expressing neurons and tested the courtship of various males toward virgin females in single pair mating experiments. Indeed, males expressing TNT in *Gr68a*-positive neurons performed poorly (Figures 2 and 3): first, an average of 41% of males failed

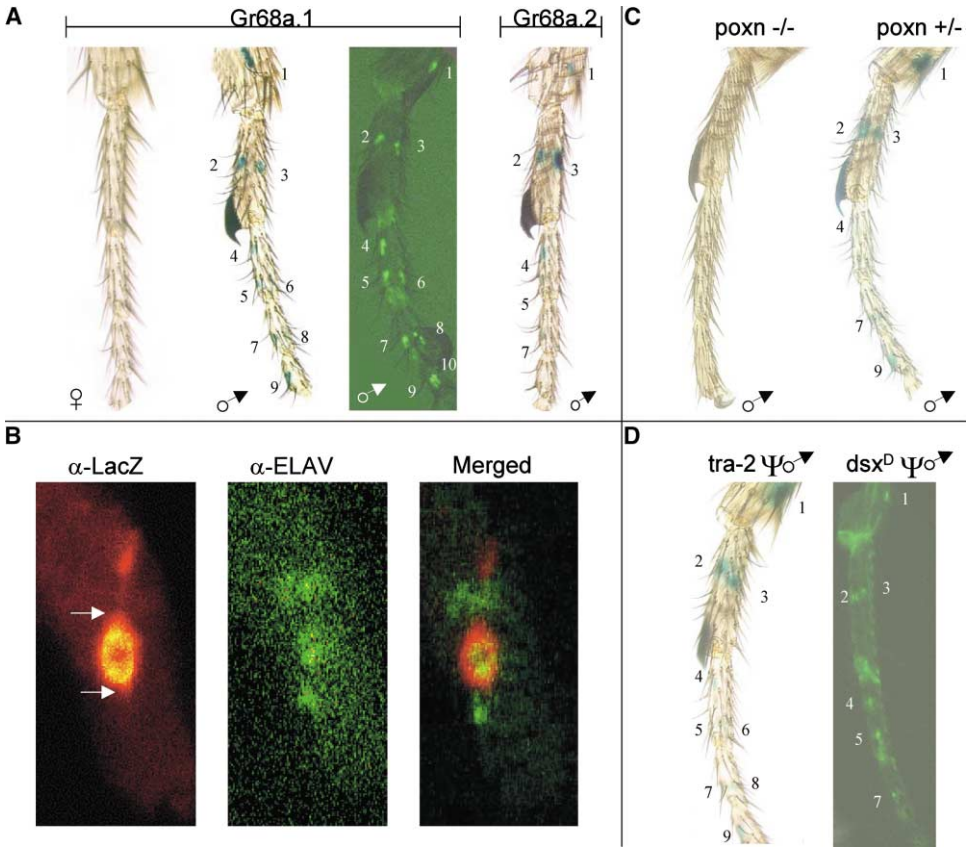


Figure 1. *Gr68a* Is Expressed in GRNs of Male-Specific Taste Bristles in the Foreleg

(A) *Gr68a* is male-specifically expressed in the foreleg. β-gal staining and GFP visualization of female and male forelegs in flies of the genotypes *p[Gr68a.1/2]-Gal4;UAS-LacZ* and *p[Gr68a.1]-Gal4;UAS-nuc-GFP*. Note that no β-gal staining is observed in the forelegs of females (left), while eight to ten β-gal- or GFP-positive cells are readily identified in forelegs of males. Also note that two independent transgenic lines, *p[Gr68a.1]-Gal4* and *p[Gr68a.2]-Gal4*, show the same expression pattern.

(B) *Gr68a* is expressed in neurons. Confocal photomicrographs of three neurons of a male foreleg, probed with an anti-ELAV antibody and an anti-β-GAL antibody and visualized with goat anti-mouse ALEXA 488 and goat anti-rabbit CY3 secondary antibody, respectively. Only the central ELAV-positive cell stains also for β-gal.

(C) *Gr68a* is expressed in chemosensory neurons. β-gal staining of forelegs from *poxn* homozygous (left) and heterozygous mutant (right) males are shown. In *poxn* mutants, chemosensory neurons are transformed into mechanosensory neurons, evident by the transformation of curved chemosensory bristles into straight mechanosensory bristles. No expression of *Gr68a* is observed in homozygous mutant *poxn* male forelegs, verifying that *Gr68a* is expressed in chemosensory and not mechanosensory neurons.

(D) Expression of *Gr68a* is regulated by the sex-determination genes. β-gal staining and GFP visualization of *Gr68a* expression in various pseudo-males (Ψ). Chromosomally female (XX) flies homozygous for a *tra-2* mutation (left) or hemizygous for the constitutive dsx^m-expressing allele, dsx^D, both of which are sexually transformed into pseudo-males. Expression of *Gr68a* in such pseudo-males is undistinguishable from wild-type males.

to mate during the observation period (30 min), whereas less than 10% of four different classes of control males were nonmaters (Figure 2, top). In addition, the males that did mate showed a significant increase in mating latency, when compared with all control males (12–14.5 min versus 6–7 min; Figure 2, bottom).

To test whether the reduction in mating performance was specifically mediated by neurons expressing *Gr68a*, we generated males in which TNT was expressed in other sets of gustatory neurons, those expressing *Gr66a* or *Gr22e*. These two *Gr* genes are expressed in a similar or larger numbers of neurons than *Gr68a*; *Gr66a* is expressed in about 14–16 neurons in the labelum and in 2 neurons of each foreleg (Dunipace et al., 2001; Scott et al., 2001); *Gr22e* is expressed in almost 100 GRNs and is the most abundantly expressed *Gr* gene characterized thus far, with extensive expression in all chemosensory organs including the legs (Dunipace et al., 2001). It should be noted that *Gr22e*, *Gr66a*, and *Gr68a* are expressed in largely (and possibly entirely) nonoverlapping groups of neurons in the foreleg (Dunipace et al., 2001). Inactivation of *Gr66a*-expressing neurons resulted in no increases in latency time and fraction of nonmaters when compared to control males (Figure 2). Males lacking functional *Gr22e*-expressing neurons exhibited a slight increase in both latency time and fraction of nonmaters when compared to wild-type or the various control males; however, this increase is only about 1/3 of that observed in males lacking *Gr68a*-expressing neurons. These experiments show that neuron identity, rather than absolute number, is important for efficient male courtship behavior and identify *Gr68a*-expressing

Table 1. Expression Profile of Different *Gr* Genes

Receptor	n (Sex)	Foreleg	Midleg	Hindleg	Wing	Labial Palps	LSO/Cibarial Organs
Gr68a.1	50 (m)	8.6	0	0	0	0	no
Gr68a.1	20 (f)	0	0	0	0	0	no
Gr68a.2	50 (m)	6	0	0	0	0	no
Gr68a.2	20 (f)	0	0	0	0	0	no
Gr66a	30	1.6	0	0	0	8	yes
Gr22e	60	4	8.2	5.7	9.8	15	yes

Expression was analyzed using *p[Gr]-Gal4;UAS-LacZ* or *p[Gr]-Gal4;UAS-nuc-GFP*. Number of β-gal- or nuc-GFP-positive cells of *Gr66a* and *Gr22e* was determined in Dunipace et al. (2001). *Gr68a.1* and *Gr68a.2* represent two independent transgenic lines of the *p[Gr68a]-Gal4* construct. *Gr68a* expression in adult flies is found exclusively in male forelegs. n indicates number of animals analyzed. Note that expression in *Gr68a.1* is more robust than *Gr68a.2*, i.e. β-gal staining is stronger after applying the exact same staining protocol. Thus, lower cell count in *Gr68a.2* might be partly or completely due to failure to score weakly positive β-gal or nuc-GFP cells.

neurons as a set of gustatory neurons critically involved in this behavior.

To test whether the reduction in mating performance of males lacking functional *Gr68a*-expressing neurons was due to reduced courtship intensity, we determined the courtship index (CI) of several types of males (for details, see Experimental Procedures). These experiments showed that males lacking functional *Gr68a*-expressing neurons spent significantly less time courting a female (CI of 40 ± 3), when compared with both groups of control males (66 ± 3 and 80 ± 3, respectively; Figure 3A). Thus, loss of mating efficiency in males lacking functional *Gr68a*-expressing neurons is caused, at least in part, by reduced courtship intensity.

Neurons Expressing GR68a Provide a Competitive Advantage in Courtship

In *Drosophila*, mating is essentially characterized by a "first come first serve basis": once fertilized, females increase egg laying and exhibit a strong rejection behavior toward additional suitors and elicit less courtship activity for several days (Hihara, 1981; Spieth and Ringo, 1983). Thus, males carrying out the courtship sequence more efficiently and flawlessly have a competitive mating advantage. In order to investigate the role of neurons expressing *Gr68a* in a more natural mating environment, we carried out three sets of competition experiments. In the first two experiments, a male lacking functional *Gr68a*-expressing neurons was forced to compete for a virgin female against either a wild-type male or a male expressing TNT^{in}. As expected, males lacking functional *Gr68a*-expressing neurons were far less successful to copulate than the competing control males under these mating conditions (only once in 46 and 40 competition experiments, respectively). The third set of competition experiments pitted the two control types, wild-type males versus those expressing TNT^{in}, against each other and showed that they had similar success rates (24 times and 16 times, respectively, in 40 experiments). Thus, these experiments established that males lacking functional *Gr68a*-expressing GRNs in each of the two forelegs performed poorly in a controlled competitive mating environment, demonstrating an essential role for these neurons in efficient male courtship behavior.

GR68a-Expressing Neurons Function in Recognition of a Female Pheromone

The results described thus far suggest that *Gr68a*-expressing neurons are involved in the recognition of a stimulatory female pheromone. A priori, however, we

Table 2. Males Lacking Functional *Gr68a*-Expressing Neurons Show Normal Geotaxis and Sugar Sensitivity

	Wild-Type	p[Gr68a.1]-Gal4; UAS-TNT^{in}	p[Gr68a.1]-Gal4; UAS-TNT
Sugar discrimination			
2 mM suc versus 20 mM tre	0.28 ± 0.06	0.30 ± 0.08	0.45 ± 0.03
2 mM suc versus 105 mM tre	0.88 ± 0.09	0.87 ± 0.03	0.92 ± 0.02
Sugar sensitivity			
0.2mM suc versus water	0.49 ± 0.07	0.54 ± 0.03	0.61 ± 0.01
5 mM tre versus water	0.67 ± 0.04	0.69 ± 0.07	0.70 ± 0.01
Geotaxis			
Gravity maze (gravity score)	179 ± 8	167 ± 17	137± 8

Males expressing tetanus toxin in *Gr68a*-expressing neurons were subjected to a series of behavioral assays along with control males. In the geotaxis assay, groups of 30–50 males were tested for their ability to sense gravity. A score of 100 indicates that all flies reach the lowest of eight vials in the maze, and a score of 400 indicates that all males reach the highest vial in the maze. Sugar discrimination and sensitivity were measured by the two choice feeding preference assay (Dahanukar et al., 2001; Ueno et al., 2001). The preference index indicates the fraction of flies that prefer trehalose; for example, a PI of 0.28 indicates that 28% of the flies fed on trehalose and 72% fed on sucrose. ±SEM. No statistically significant differences among the genotypes tested (ANOVA; $p > 0.05$) were observed for sugar sensitivity, discrimination, or geotaxis. For details on assays, see Experimental Procedures.

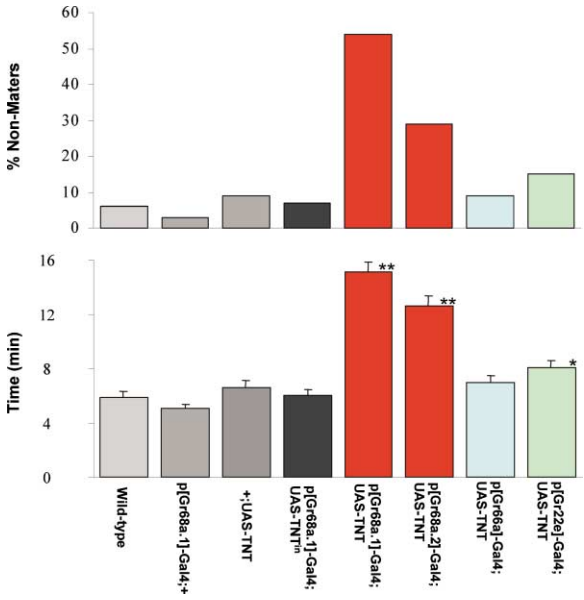


Figure 2. Reduced Courtship Behavior of Males Expressing Tetanus Toxin in Gr68a-Positive GRNs

The top diagram shows the percentage (n = 100 for each genotype) of males that fail to copulate within the 30 min observation period. The bottom diagram shows average copulation latency (see Experimental Procedures). Control males (males containing only the driver or the reporter, males expressing TNT^{in}, and *Ore-R* wild-type males) are shown in shades of gray, males of two independently created lines expressing tetanus toxin in *Gr68a*-positive neurons are shown in red, and males expressing tetanus toxin in *Gr66a*- or *Gr22e*-positive neurons are shown in blue and green, respectively (see also Table 1). Note that 30%–50% of males lacking functional *Gr68a*-expressing neurons fail to mate compared to less than 10% of control males. Error bar represents ±SEM. Statistical analysis: the four control genotypes (gray) were combined into one group, as initial ANOVA showed no statistical difference ($p > 0.05$) in latency time. ANOVA analysis for the combined control group and the four experimental groups revealed statistically significant differences between groups ($p < 0.0001$); subsequent LSD comparison showed that the two lines expressing TNT in *Gr68a*-positive neurons had a highly significant delay in latency time (**$p < 0.01$), while TNT in *Gr22e*-positive neurons caused a less significant delay (*$p < 0.05$). No statistically significant delay was observed in males expressing TNT in *Gr66a*-positive neurons.

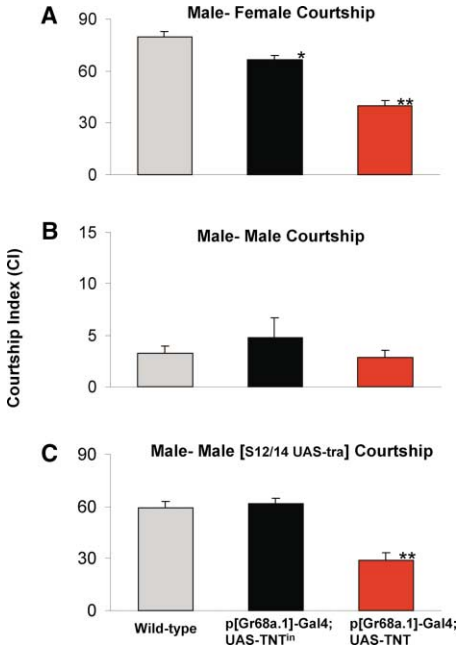


Figure 3. Courtship Index of Males Lacking Functional *Gr68a* Neurons Is Reduced in Male-to-Female but Not Male-to-Male Courtship

(A) The courtship index (CI) of various males courting virgin females was determined during a 10 min observation period (n = 30 for each genotype tested). The CI is given as a fraction of the time that a male spends performing any of the five steps of the courtship sequence prior to copulation (i.e., an index of 40 indicates that a male spends 4 of the 10 min courting the female). Error bars represent ±SEM. Statistically significant differences in male-female CI between the controls and the experimental lines was determined using ANOVA ($p < 0.0001$). Pairwise comparison of wild-type and experimental males showed a significantly stronger reduction in CI for *p[Gr68a.1]-gal4; UAS-TNT* (**$p < 0.0001$) than for *p[Gr68a.1]-gal4; UAS-TNT^{in}* (*$p < 0.005$) using the t test.

(B) The diagram shows the CI of various males toward target males (n = 16 for each genotype tested). Error bars represent ±SEM. All males show very little courtship activity toward other males, without any significant difference in the CI (ANOVA, $p > 0.05$).

(C) The diagram shows the CI of various males toward target males with feminized oenocytes (pheromone-producing cells) in the abdomen (these males express the feminizing TRA protein under the control of the S12/14 enhancer). Twenty flies from each genotype were analyzed. Error bars represent ±SEM. Statistically significant differences in male-male$^{S12/14;UAS\text{-}tra}$ CI between the controls and the experimental lines was determined using ANOVA ($p < 0.0001$). Pairwise comparison of wild-type and experimental males showed a significantly stronger reduction in CI for *p[Gr68a.1]-gal4; UAS-TNT* (**$p < 0.0001$), when compared to the two controls.

could not exclude the possibility that these neurons might recognize an inhibitory pheromone present in males. To investigate this possibility, we performed two sets of experiments: first, we paired males lacking functional *Gr68a*-expressing neurons, wild-type males and males expressing the inactive tetanus toxin protein with virgin control males and determined male-to-male courtship behavior by measuring their CI. This analysis revealed that all tested males exhibited virtually no courtship activity toward other males (Figure 3B), an observation that further supports a role for *Gr68a*-expressing neurons in the recognition of a stimulatory female pheromone. Second, to address the possibility that reduced courtship of males lacking functional *Gr68a*-expressing neurons could be attributed to their failure to recognize other female cues (visual, behavioral, etc.), we subjected the three types of males to single mating experiments with males exhibiting a female pheromone profile. These males (*S12/14; UAS-tra*), which are visually and behaviorally indistinguishable from wild-type males, express the TRA protein under the control of an inducible GAL4 protein in secretory cells including oenocytes (Roman et al., 2001). Selective expression of TRA in oenocytes results in the production of female pheromones in otherwise normal males, which elicit aggressive courtship from other males including wild-type males (Ferveur et al., 1997). When *S12/14; UAS-tra* males were used as mating partners for males expressing the active TNT protein in *Gr68a*-positive cells, we observed a reduction in the CI similar to that observed with female mating partners; similarly, the con-

trol males showed virtually as high of a CI toward these "pheromonally feminized" males as they do toward virgin females (Figures 3A and 3C).

Taken together, our data strongly suggest that the *Gr68a*-expressing neurons in the male forelegs are necessary for the recognition of a female pheromone component.

Gr68a-Expressing Neurons Are Required Early in the Courtship Sequence

Drosophila courtship consists of a sequence of behaviors, the proper order of which is crucial for efficient mating (Figure 4A). To identify the specific step(s) in which *Gr68a* is involved, we quantified three individual courtship steps (1, 3, and 5), all of which can be readily quantified (for details, see Experimental Procedure). The two types of control males initiated courtship about once per minute, whereas males lacking functional *Gr68a*-expressing neurons had a modest, but significant, increase in initiation (1.4 times/min, Figure 4B). Interestingly however, wing extension/vibration and attempted mating were 2- to 3-fold reduced in these males when compared to the two control males (Figure 4B). Thus, our analysis shows that the neurons expressing GR68a are crucial after step 1 and before step 3, suggesting that males without proper pheromone input through the *Gr68a*-expressing neurons in the forelegs stall at the tapping step during male courtship (Figure 4A).

GR68a Receptor Is Required for Normal Male Courtship

Expression studies of about a quarter of all *Gr* genes has revealed that, with the exception of *Gr22e*, most are expressed in a small number of GRNs (Dunipace et al., 2001; Scott et al., 2001). These studies suggested that a single GRN expresses a very small number—possibly just one—of the 70 different receptor genes. Hence, the phenotype observed in males lacking functional *Gr68a*-expressing neurons could be attributed solely to a second GR protein present in these cells. To address this possibility, we employed RNA interference to knock out/down the expression of *Gr68a* RNA/protein (Fire et al., 1998; Piccin et al., 2001). We generated males expressing a double-stranded *Gr68a* RNA (*UAS-ds_Gr68a*) under the control of the *p[Gr68a]-Gal4* driver and observed a statistically significant reduction in mating performance both with regard to the fraction of nonmaters and the increase in latency time (Figure 5). The more modest phenotype compared to males lacking functional *Gr68a*-expressing neurons might be explained by a temporal delay of *ds_Gr68a* RNA expression, which requires first the accumulation of GAL4 protein; hence some GR68a protein may be produced before *ds_Gr68a* RNA is transcribed to promote endogenous *Gr68a* RNA degradation. Alternatively, *Gr68a* RNA might not be efficiently degraded or a second *Gr* gene expressed in these neurons might partially substitute for *Gr68a* function. To investigate these possibilities, we subjected older males, aged for an additional 7–10 days, to the single mating assay. If protein turnover is the major cause for the difference between young males expressing TNT versus *ds_Gr68a* RNA, the phenotype should become more severe in older males expressing *ds_Gr68a*; if, on the other hand, incomplete knockdown or presence of a second receptor is the major cause for the weaker phenotype, no change in severity should be observed in older males. Single mating experiments revealed that older males indeed showed a further reduction in courtship performance, reaching levels similar to males lacking *Gr68a*-expressing neurons altogether (Figures 2 and 5). The CI of these males toward virgin females was also significantly reduced to 50.8 $\pm$ 4, a value close to that observed in males expressing TNT in GR68a-positive neurons (40.0 $\pm$ 3). Importantly, age per se had no effect on courtship performance because older control males showed no reduction in mating efficiency and were indistinguishable in their performance from young control males.

Finally, we also quantified individual courtship steps of *ds_Gr68a* RNA-expressing males in order to determine the courtship deficit more precisely. We found that these males showed also a significant decrease in executing later courtship steps, such as wing extension/vibration, mating attempts, as well as copulation, whereas courtship initiation was increased, just as observed in males expressing TNT (compare Figures 4B and 4C). We note that no reduction in courtship performance was observed when *ds_yellow* RNA, which reproduces an exact phenocopy of null *yellow* mutations (Piccin et al., 2001), was expressed in GR68a-positive neurons, showing that the phenotype was gene specific (Figures 4 and 5). Thus, our data strongly support the notion that GR68a expressed in male-specific neurons functions as the crucial receptor involved in the recognition of a female pheromone.

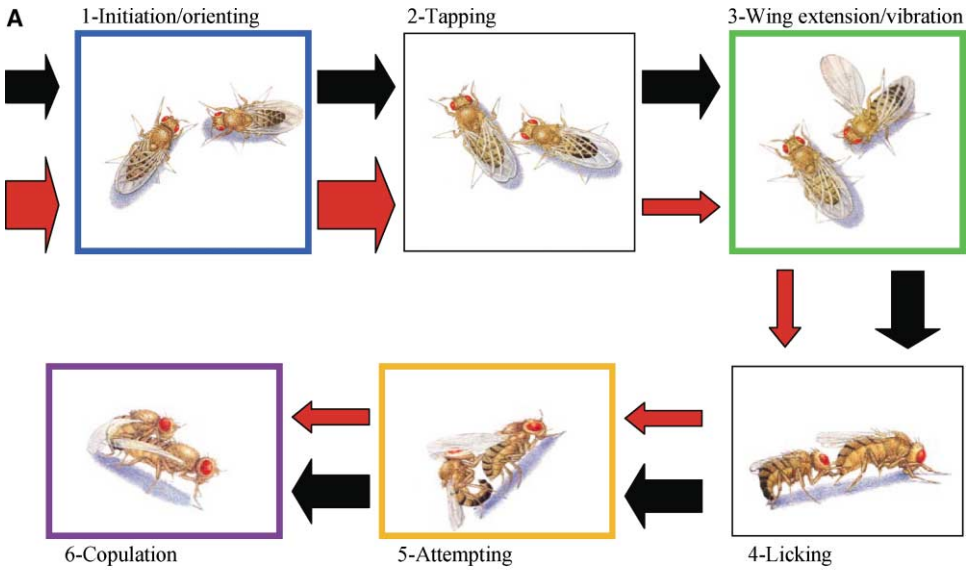
Discussion

The recognition of sex pheromones by potential mates is a universal feature in the courtship behavior of most animal species. Yet, surprisingly little is known about the specific pheromone receptors, signal transduction pathways and neuronal networks that integrate chemosensory with various other sensory inputs during courtship.

Here, we showed that a set of about 20 neurons associated with male-specific taste bristles in the forelegs of *Drosophila melanogaster* is crucially involved in pheromone recognition during male courtship behavior. These bristles are molecularly characterized by the expression of the proposed taste receptor GR68a. RNA-mediated repression of this gene showed that Gr68a is in fact directly involved in recognition of a female pheromone, providing a precedent for a sex-specific pheromone receptor with a defined function in courtship behavior.

Dissection of a Complex Behavior

In principle, courtship behaviors serve two purposes: to attract the attention of a mating partner and to identify the sex and mating status of a con-specific animal. The complex sequence of behaviors of male *Drosophila melanogaster* combines both these purposes and is critical in guiding the male in a coordinated fashion through the entire courtship ritual culminating in successful copulation. The perception of female pheromones during the



B

	Initiation per minute	Wing ext/vib per minute	Attempting per minute	Copulation per 30 minutes
Wild-type	0.8 ± 0.1	7.9 ± 0.4	5.2 ± 0.3	94%
p[Gr68a.1]-Gal4; UAS-TNT^{in}	1.0 ± 0.1	6.8 ± 0.4	4.2 ± 0.4	93%
p[Gr68a.1]-Gal4; UAS-TNT	1.4 ± 0.1**	3.8 ± 0.2**	1.8 ± 0.1**	46%

C

p[Gr68a.1]-Gal4; UAS-ds_yellow	0.9 ± 0.1	8.2 ± 0.4	3.1 ± 0.2	94%
p[Gr68a.1]-Gal4; UAS-ds-Gr68a	1.1 ± 0.1*	4.2 ± 0.3**	2.0 ± 0.2**	69%

Figure 4. *Gr68a*-Expressing Neurons Are Required during the Tapping Step

(A) Model for step-to-step transition during male courtship behavior in wild-type males and males lacking Gr68a receptor/neurons. The male (darker pigmented abdomen) is on the right in top three panels (steps 1–3) and on the left in panels at the bottom (step 4–6). The arrows indicate transition from each step to the next. The normal transition from one step to the next observed in wild-type and control males is indicated by the constant thickness of the black arrows; the altered thickness of the red arrows indicates the changes in these transitions in impaired males, which either expressing TNT or *ds_Gr68a* RNA. Drawings adapted from Greenspan (1995).

(B) Individual courtship steps were quantified in males with functional (wild-type, *TNT^{in}*) and inactivated (*TNT*) *Gr68a*-expressing neurons by measuring the initiation, wing extension/vibration, and copulation attempts during the first 5 min of a single pair mating (or until copulation occurs; for details on assay, see Experimental Procedures). The mating frequency reflects percentage of males that copulate within 30 min (see Figure 2). Note that initiation/orientation is significantly increased in males with inactive *Gr68a*-expressing neurons when compared to the two control males, whereas later steps (3, 5, and 6) showed a significant reduction (2-fold). ANOVA analysis ($p < 0.0001$) and pairwise comparison (t test) showed statistically significant differences between *p[Gr68a.1]-Gal4; UAS-TNT* and the two controls (*p[Gr68a.1]-Gal4; UAS-TNT^{in}* and wild-type; **$p < 0.005$).

(C) Individual courtship steps were quantified in males expressing *ds_Gr68a*RNA or *ds_yellow* RNA under the control of the *p[Gr68a]-Gal4* driver, measuring the initiation, wing extension/vibration, and copulation attempts during the first 5 min of a single pair mating (or until copulation occurs; for details on assay, see Experimental Procedures). The mating frequency reflects percentage of males that copulate within 30 min (see Figure 5). As in males with inactivated *Gr68a* neurons (see Figure 4B), initiation/orientation is significantly increased in males expressing *ds_Gr68a*RNA when compared to control males expressing *ds_yellow* RNA, whereas later steps (3, 5, and 6) showed a significant reduction (2-fold). Pairwise comparison (t test) showed statistically significant differences between *p[Gr68a.1]-Gal4; UAS-ds_Gr68a*RNA and *p[Gr68a.1]-Gal4; ds_yellow* control males (*$p < 0.05$; **$p < 0.005$).

second and fourth step of the sequence are crucial events of courtship and must be integrated with other sensory input, including visual cues (female-specific coloration of the abdomen) and behavioral responses of the female toward the male during the entire courtship.

The functional characterization of the *Gr68a*-expressing neurons associated with male-specific taste bristles of the forelegs provided an opportunity to dissect the male courtship behavior. When *Gr68a*-expressing neurons were functionally inactivated by coexpressing TNT, males showed a significant reduction in courtship activity toward females or males with a female pheromone profile, but no increase of courtship toward other males (Figures 2 and 3). Therefore, these neurons mediate a stimulatory response of an attractive, female pheromone as opposed to a repressive response of an inhibi-

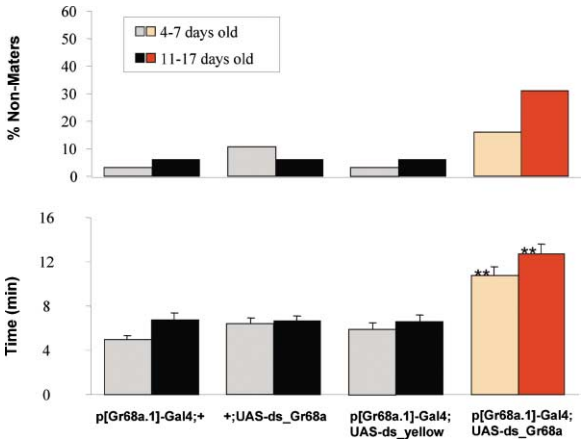


Figure 5. Knockdown of *Gr68a* Expression by RNAi Leads to an Increase in Latency Time and Fraction on Nonmaters

The top diagram shows the percentage ($n > 59$ for each age/genotype) of males that fail to copulate within the 30 min observation period. The bottom diagram shows average copulation latency (see Experimental Procedures). Control males (males containing only the driver, the reporter, or *p[Gr68a.1]-Gal4; UAS-ds_yellow*) are shown in black/gray; males expressing *ds_Gr68a* RNA under the control of *p[Gr68a.1]-Gal4* are shown in red/orange. All mating experiments were carried out with 4- to 7- (gray and orange) or 11- to 17- (black and red) day-old males, respectively. Error bars represent ±SEM. Statistically significant differences between the controls and the experimental lines was determined using ANOVA ($p < 0.0001$). Post hoc LSD test showed that both young and old *p[Gr68a.1]-Gal4;UAS-ds_Gr68* males had a significant increase in mating latency compared to the controls (**$p < 0.01$). Note that latency time and fraction of nonmaters in old males is increased compared to young males; older age, however, had no effect on mating latency or the fraction of nonmaters in any of the control males.

tory male pheromone. The specific role for these neurons was associated with the tapping step during courtship (step 2), in which the male directly contacts the pheromone-coated abdomen of the female with the tarsi of his forelegs (Greenspan, 1995; Greenspan and Ferveur, 2000; Hall, 1994). By quantitatively analyzing individual courtship steps, we showed that males lacking function *Gr68a*-expressing neurons stall during the second step (Figures 4A and 4B). Moreover, the modest increase in initiation/orientation (step 1) suggests that these males "start over" more often with the courtship sequence than males with intact *Gr68a*-expressing neurons. Knowing the identity of these neurons and the specific phenotype associated with their inactivation should provide future opportunities to address more complex questions pertinent to this intriguing behavior. For example, how is the pheromone input in step 2 integrated with visual information received during step 1 and additional pheromone input received in step 4? And how does this input affect the motorneuron output so strikingly displayed in step 3 and 5 (Figure 4A)? At least a partial answer to these questions will require the identification of the first- and second-order target neurons of the *Gr68a*-expressing sensory neurons, which eventually should become feasible using axonal, synaptic, and *trans*-synaptic marker proteins expressed under the control of the *Gr68a* promoter.

The GR68a Receptor Is Essential for Efficient Courtship

The courtship phenotype associated with inactivating Gr68a-expressing neurons is likely to be mediated by the GR68a receptor itself. Males in which GR68a expression was suppressed by RNAi had an increase in mating latency, fraction of nonmaters, and reduced courtship intensity, as was observed in males in which the *Gr68a*-expressing neurons were inactivated altogether (Figure 5). Moreover, the detailed courtship analysis revealed that these males also stall during the same step in the courtship sequence, with almost identical severity as males with inactivated *Gr68a*-expressing neurons, arguing for a major role of this receptor in recognition of a female pheromone (compare Figures 4B and 4C). It is quite possible that *Gr68a* is the only *Gr* gene that is expressed in these neurons. Reported expression studies of about ten *Gr* genes (Dunipace et al., 2001; Scott et al., 2001) and ongoing studies of an additional ten *Gr* genes (N. Thorne and H.A., unpublished data) indicate that most Gr genes are expressed in distinct sets of gustatory neurons, and expression of different *Gr* genes are to a large extend nonoverlapping. In any case, even if *Gr68a*-expressing neurons coexpress another *Gr* gene, its transcripts are unlikely to be affected by the expression of *ds_Gr68a* RNA, because nucleotide sequence similarity between *Gr68a* and any other *Gr* gene is far too low to allow RNAi to occur.

To our knowledge, *Gr68a* is the first putative, sex-specific, pheromone receptor gene with a defined function in courtship behavior. A gene cluster containing 16 putative pheromone receptors (V1Rs) expressed in the vomeronasal organ (Dulac and Axel, 1995) was recently reported to be required for normal mating behavior of mice (Del Punta et al., 2002). Female mice homozygous for this multigene knockout showed reduced aggression toward invaders, and homozygous male mice showed reduced sexual aggression toward both sexes. However, none of the *V1Rs* included in that deletion were reported to be sex specific, and it remains to be investigated whether specific behavioral phenotypes can be associated with individual *V1R* genes.

Diverse Roles for the GR Proteins

The 70 *Drosophila Gr* genes, which are distantly related to the *olfactory receptor* (*Or*) genes, encode a diverse family of G protein-coupled receptors that share between 15% and 80% sequence similarity. No other candidate chemosensory receptors have emerged from the complete genome sequence of *Drosophila*, suggesting that the GR proteins might accommodate the detection of all nonvolatile substrates to which *Drosophila* is responsive. In mammals, distinct groups of nonvolatile compounds are recognized by unrelated G protein-coupled receptors, encoded by four distinct gene families that are expressed in neurons of the vomeronasal organ or in taste cells of the tongue, respectively (reviewed by Dulac, 2000; Montmayeur and Matsunami, 2002). For example, the taste cells in the tongue express two classes of receptors, the T1Rs and T2Rs; the T1Rs were shown to detect sweet-tasting substrates such as various sugars and many L-amino acids (Max et al., 2001;

Nelson et al., 2001), whereas the much more numerous T2Rs appear to recognize the large spectrum of compounds perceived as bitter tasting to humans (Chandrashekar et al., 2000).

The only known substrate for a GR protein, GR5a, is trehalose (Dahanukar et al., 2001; Ueno et al., 2001), which is an important food source for *Drosophila melanogaster*. The number of biologically relevant sugars is fairly small compared to the number of *Gr* genes, and hence, members of this protein family are likely to recognize other classes of substrates. We suggest that GR68a is a receptor that detects a pheromone(s) of *Drosophila melanogaster* females, possibly a long-chain hydrocarbon such as the female-specific 7,11 heptacosadiene and 7,11 nonacosadiene, both of which have been strongly implicated in eliciting male courtship behavior (Coyne and Oyama, 1995; Ferveur et al., 1996). Thus, we propose that the GR protein family recognizes a whole spectrum of nonvolatile, complex substrates (sugars, amino acids, alkaloids and bitter-tasting compounds, hydrocarbons, and possible other pheromones, etc.) to which *Drosophila* responds. For example, different sugars might bind to receptors of the GR5a subfamily, which include seven additional receptors encoded by *Gr61a* and *Gr64a-f* (members of a subfamily were defined by sharing at least 34% sequence similarity [Dunipace et al., 2001]). Similarly, proteins encoded by the *Gr68a* subfamily—*Gr2a, Gr32a* and *Gr39a.a-39a.d*—might interact with different pheromone components. Expression of *Gr32a* has been analyzed and found to be restricted to the labelum and the distal tip of the forelegs of both sexes (Scott et al., 2001). Preliminary experiments indicate that simultaneous inactivation of *Gr68a*- and *Gr32a*-expressing neurons by TNT compounds the courtship defect in males, resulting in about 80% nonmaters and almost 20 min latency time, suggesting that this receptor might function in the fourth step of the courtship sequence (S.B. and H.A., unpublished data). Further analysis of *Gr32a* and the other members of the *Gr68a* subfamily using RNAi should reveal the specific roles, if any, that these genes have during male mating behavior.

Finally, amino acids and various classes of bitter-tasting substrates might be recognized by receptors encoded by yet other *Gr* subfamilies. It is even conceivable that some GR proteins detect volatile molecules, as a few *Gr* genes were found to be expressed in olfactory neurons both in the larvae and the adult (Dunipace et al., 2001; Scott et al., 2001). Thus, the highly diverse GR proteins are likely to mediate a multitude of strikingly different behaviors including courtship and mating, feeding behavior, and avoidance behavior elicited both by soluble and volatile compounds.

Experimental Procedures

β-Gal Staining and GFP Visualization

For whole-mount β-gal staining, flies were fixed for 30–40 min in 1% paraformaldehyde in 1×PBS, washed briefly in 1×PBS, and then placed in X-gal buffer (0.1 M sodium phosphate [pH 7.4], 2 mM magnesium chloride, 0.01% sodium deoxycholate, 0.02% Nonidet p40). Flies were dissected in X-gal buffer and then stained overnight at 37°C on a rotating platform in X-gal staining solution (X-gal buffer with 1 mg/ml X-gal, 1 mM spermidine HCl, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide). On day 2, the tissue was rinsed in 1×PBS and then stored in 70% glycerol in 1×PBS. Stained tissue was mounted on slides and analyzed using a Zeiss Axioskop 2 microscope. For live GFP visualization, anesthetized flies were dissected and mounted on slides in 70% glycerol in 1×PBS. GFP was visualized using a Zeiss Axioskop 2 microscope.

Antibody Staining

Fly legs were embedded in OCT compound and sectioned (12 um) using a Reichert-Jung cryostat. Tissue was fixed for 7 min in 2% paraformaldehyde in 1×PBS then washed two times for 10 min in 1×PBS. Leg sections were permeabilized for 30 min in PT (1×PBS, 0.1% Triton X-100) and then blocked for 30 min in PTS (PT with 5% heat-inactivated goat serum). Following blocking, sections were incubated overnight at 4°C with anti-β-galactosidase antibody (1:1000; Cappel/ICN, Aurora, OH) and anti-ELAV antibody (1:10) diluted in PTS. The next day the tissue was washed five times for 10 min in PT, again blocked for 30 min in PTS, and then incubated for 2 hr at room temperature with goat anti-rabbit CY3 (1:500; Jackson Immuno Research, West Grove, PA) and goat anti-mouse ALEXA 488 (1:100; Molecular Probes, Eugene, OR) in PTS. After washing in PT, the slides were mounted with ProLong Antifade Kit (Molecular Probes) and analyzed with a Zeiss LSM 410 confocal microscope (Thornwood, NY).

Transgenes and Transformation of *Drosophila* Embryos

p[Gr68a]-Gal4

A 3.4 kb fragment just upstream of the translation initiation codon was amplified from genomic DNA using specific primers and cloned into pGEM-T Easy vector from Promega (Madison, WI) and sequenced. This fragment was excised by BsiWI/NotI and cloned into the Acc65/NotI sites of the Gal4 transformation vector SM1, and transgenic flies were generated as described previously (Dunipace et al., 2001).

UAS-ds_Gr68a

The coding region of *Gr68a* was amplified from genomic DNA using gene-specific primers, cloned, and sequenced in the pGEM-T Easy vector. The *Gr68a* coding sequence was subcloned into the pEGFP-N3 vector from Clontech (Palo Alto, CA) using the HindIII and SalI restriction sites. The *Gr68a-Gfp* sequence and *Gr68a* coding sequence were then sequentially subcloned into the pUAST (Brand and Perrimon, 1993) vector, to create *UAS-ds_Gr68a* in which the sense orientation and antisense orientation of *Gr68a* were separated by the coding sequence of *Gfp,* serving as a spacer. Details on PCR and subcloning are available upon request.

Genetics

The following fly strains were used for *Gr68a* expression analysis: (1) w^{1118}, (2/3) *p[Gr68a.1/2]-Gal4*, (4) *w;UAS-lacZ*, (5) *w;UAS-nuc-gfp*, (6) *w;CyO/UAS-LacZ poxn*$^{70\text{-}23}$, (7) *w;CyO/42WMG; p[Gr68a.1]-Gal4*, (8) B^sY*/w; CyO/tra2; UAS-LacZ*, (9) B^sY*/w; CyO/tra2;p[Gr68a.1]-GAL4*; (10) B^sY*; dsxD Sb/TM6/Df(dsx)*, and (11) *w; CyO/UAS-nucGfp; p[Gr68a.1]-Gal4 Df(dsx)/TM3 Sb*,

The following fly strains were used for behavioral analysis: (12) *UAS-tnt*; (13) *UAS-tnt*^{in}, (14) *w;p[Gr66a]-Gal4*; (15) *w;p[Gr22e]-Gal4*, (16) *w;UAS-ds_Gr68*; (17) *Oregon-R* (wild-type), and (18) *S12/14*. The genotypes depicted in this paper were obtained by a single cross of the corresponding strains.

Behavioral Assays

Statistical Analysis

Statistical analysis of all behavioral assays was done using analysis of variance (ANOVA) of groups and post hoc LSD and t test. For details see figure and table legends.

Courtship Behavior

All males used in mating assays were of w^+ background to eliminate any effects of different eye pigmentation on mating performance. Female and male mating targets were from the w^{1118} strain, unless noted otherwise. Males and females were collected as virgins shortly after eclosion. Males were kept in isolation until used for mating behavior assays at the age of 4–7 days, unless indicated otherwise;

females were kept in groups of 10–40 flies until used as mating targets 2–5 days old. All flies were kept at 25°C on 12:12 light:dark cycle. Mating experiments were performed in late afternoon in artificial daylight in a small Plexiglas mating chamber (4 × 10 × 30 mm). Copulation latency was measured from the time a single male was aspirated into the mating chamber until he successfully mounted the female. A nonmater was a male that did not copulate within 30 min. The courtship index (CI) is given as percentage of $T_{[courting]}/T_{[total]}$, whereby $T_{[courting]}$ is defined as time a male performs one of the five courtship behaviors (orienting, tapping, wing vibration, licking, and attempting) and $T_{[total]}$ is defined as the time until copulation occurs or 10 min (observation period) if no copulation occurred during a 10 min observation period (Hall, 1994). Competition experiments were performed by placing two males in a chamber with a single female and scoring the male which successfully mated. One of the males was marked with a dot of blue ink on the thorax shortly after eclosion at the time of collection in order to distinguish between genotypes. The blue dot did not affect courtship vigor (data not shown).

Quantification of individual courtship steps was carried out in two series of experiments: in the first, we counted the number of times a male initiated the courtship sequence toward a mating target and the number of wing extensions/vibrations a male performed toward that target. An initiation (step 1) was scored when a male approached its mating target to a distance equal or less than a head diameter, following 3 s or more of no mating activity. Thirty animals of each genotype were analyzed. In the second series of experiments, we counted the number of wing extensions/vibrations and the number of attempts at copulation (bending the abdomen in an angle of 90 or more degrees). Fifteen animals of each genotype were analyzed. Each experiment lasted for 5 min or until mating occurred. The investigator was unaware of the genotype of the mating subject at the time of the experiment to avoid any subjective bias.

Two-Choice Feeding Preference Test

Feeding preference was essentially carried out as described in (Dahanukar et al., 2001). Lowest concentration of sucrose and trehalose were determined by stepwise reduction of each substrate and comparison to water.

Geotaxis Behavior

Geotaxis assay was performed as described in Toma and coworkers (Toma et al., 2002) with the following modifications: up to 60 males were introduced at the origin of the maze which consisted of clear plastic tubing that bifurcated three consecutive times in vertical direction to generate eight destination points that were hooked up to food vials. Each vial was given a value between 1 and 4, depending on the number of down decisions that were necessary to reach a vial (1 = 3 times down, 2 = twice down/once up, 3 = once down/twice up, and 4 = three times up). The "weighted values" of each vial were determined (percentage of flies in each vial multiplied by the value of the vial) and summed up to generate the geotaxis value, v_g. A v_g of 100 indicates that all flies in the maze reached the lowest vial, whereas a v_g of 400 indicates that all flies in the maze reached the top vial. Flies that did not reach the tubing beyond the last bifurcation were discarded (less than 5%).

Further details on behavioral experiments are available upon request.

Acknowledgments

We thank Drs. Lawrence Katz and Hiroaki Matsunami for suggestions during the course of this work; Drs. Jeff Hall, Raquel Sitcheran, Lawrence Katz, and Hiroaki Matsunami provided useful comments on the manuscript. We thank Philippe Luedi for help with statistical analysis and Sean Sweeney, Leslie Vosshall, and Gregg Roman for fly strains. This work was supported by a grant from NIH to H.A.

Received: February 13, 2003
Revised: May 9, 2003
Accepted: August 11, 2003
Published: September 10, 2003

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